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**Noninvasive assessment of liver disease severity with liver fat score and CK-18 in NAFLD: Prognostic value of liver fat equation goes beyond hepatic fat estimation.**

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with respect to wild-type animals fed water, and the enrichment with fructose exclusively caused the restoration of the significantly increased levels of these two parameters. These results clearly suggest that the presence of TLR-4 is essential to explain liver damage, body weight gain, and ALT impairment due to the fructose intake.

Furthermore, the authors found that plasma endotoxin levels were significantly increased both in wild-type and mutant mice fed chronically with a 30% fructose solution, in comparison to water-fed controls.

The role of fructose in NAFLD development was not entirely unknown to researchers. In particular, a recent work<sup>4</sup> demonstrates that patients with NAFLD have a significantly greater consumption of fructose than controls, and an increased hepatic expression of fructokinase messenger RNA. Although the role of TLR-4 in carbohydrate-dependent NAFLD has been only recently suggested by Thuy and colleagues,<sup>5</sup> they have pinpointed one of the potential mechanisms through which fructose could participate in NAFLD development and progression in humans: a carbohydrate-rich diet may produce ethanol when intestinal stasis favors bacterial overgrowth in the upper parts of the gastrointestinal tract. The increased portal endotoxemia could initiate TLR signaling and induce necroinflammation, which characterizes steatohepatitis, the most advanced form of NAFLD. Accordingly, this study also highlighted significant correlations between hepatic expression of TLR-4, plasminogen activator inhibitor 1, and endotoxin, even though they are still unable to explain the molecular signaling pathways.

Interestingly, another recent study investigated the potential importance of Kupffer cells and TLR-4 in the pathogenic mechanisms underlying nonalcoholic steatohepatitis induced by a methionine-deficient and choline-deficient diet.<sup>6</sup>

Unfortunately, the study by Spruss et al. does not provide additional clues to the mechanisms by which fructose intake, endotoxemia, and the resulting activation of TLR-4 signaling might promote NAFLD. On the other hand, the experimental results in this work allow the exclusion of the involvement of some important TLR-4-dependent proinflammatory inducing transcriptional factors (i.e., IRF3 and IRF7), suggesting that fructose feeding may lead to NAFLD through an insulin-independent *de novo* lipogenesis and/or an endotoxin-dependent activation of Kupffer cells. In this last hypothesis, an interaction network which involves TLR-4, Myd88, c-Jun N-terminal kinase, and nuclear factor  $\kappa$ B might induce tumor necrosis factor- $\alpha$  production and release, oxidative stress, and insulin resistance.<sup>7</sup>

We believe that although Spruss et al. present a well-conducted study, the precise role of TLR-4-dependent pathways in NAFLD requires further experimentation. In fact, it is possible that new additional signaling proteins of innate immunity, as yet uncovered, may be involved in the necroinflammatory process and in the progression to steatohepatitis and fibrosis.

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## Noninvasive Assessment of Liver Disease Severity With Liver Fat Score and CK-18 In NAFLD: Prognostic Value of Liver Fat Equation Goes Beyond Hepatic Fat Estimation

### To the Editor:

We read with interest the article by Kotronen et al.,<sup>1</sup> reporting on liver fat score and liver fat equation—new noninvasive, easy-to-calculate indexes that estimate the presence and severity of hepatic fat accumulation. We assessed the ability of these two indexes and of plasma cytokeratin-18 fragments (CK-18) to predict the presence of nonalcoholic fatty liver disease (NAFLD) and of nonalcoholic steatohepatitis (NASH),<sup>1,2</sup> respectively, and their relations to validated predictors of incident cardiovascular disease and diabetes.<sup>3,4</sup>

To this purpose, 125 subjects (40 nondiabetic patients with biopsy-proven NAFLD and 85 healthy controls) underwent an oral fat tolerance test,<sup>5</sup> with measurement of postprandial plasma lipid responses, and a standard oral glucose tolerance test (OGTT), whose results were elaborated by Minimal Model analysis to assess whole-body, hepatic, and muscle insulin sensitivity and indexes of pancreatic  $\beta$ -cell function

(namely, CP-genic index [CGI] and Adaptation Index [AI]), as previously described.<sup>5-7</sup> Finally, circulating markers of inflammation (C-reactive protein), endothelial dysfunction (E-selectin and intercellular adhesion molecule-1 [ICAM-1]) and oxidative stress (nitrotyrosine and oxidized low-density lipoproteins) were measured.

Results are shown in Table 1. NASH group showed higher postprandial lipemia and oxidative stress than either steatosis or controls. Patients with NASH had also more severe whole-body insulin resistance, hepatic insulin resistance, and pancreatic  $\beta$ -cell dysfunction and higher plasma C-reactive protein, E-selectin, ICAM-1, and nitrotyrosine levels than steatosis and control groups.

Liver fat equation correlated with the degree of histological steatosis in both NASH and steatosis groups (in both groups:  $r_s > 0.66$ ,  $P < 0.003$ ). The area under the receiver operating characteristic curve (AUROC) of liver fat score for predicting NAFLD was 0.86 (95% confidence interval [CI]: 0.82-0.91). A cutoff of  $-0.640$  individuated

**Table 1. Baseline Characteristics of Patients with NAFLD and Controls**

Characteristic	Controls (n = 85)	Steatosis (n = 17)	NASH (n = 23)	P Value
Age (years)	49 ± 4	48 ± 3	46 ± 4	0.712
Sex (%males)	67	70	71	0.549
BMI (kg/m <sup>2</sup> )	25.1 ± 1.7	24.9 ± 1.6	25.3 ± 1.5	0.656
Systolic BP (mmHg)	129 ± 6	130 ± 7	131 ± 9	0.978
Diastolic BP (mmHg)	81 ± 4	84 ± 6	88 ± 6*	0.034
Waist (cm)	88 ± 2	90 ± 4	91 ± 4	0.478
Tg (mg/dL)	89 ± 21	98 ± 35	111 ± 39*	0.112
LDL-C (mg/dL)	99 ± 7	124 ± 8	150 ± 7*	0.056
HDL-C (mg/dL)	59 ± 2	57 ± 2	49 ± 2*	0.013
Total C (mg/dL)	156 ± 9	205 ± 9	241 ± 10†	0.058
Glucose (mg/dL)	92 ± 3	96 ± 3	97 ± 4	0.541
Insulin (μU/mL)	4.2 ± 1.6	14.3 ± 2.9†	22.7 ± 5.6†	0.009
AST (U/L)	13 ± 3	56 ± 3†	42 ± 3†	0.123
ALT (U/L)	16 ± 5	127 ± 8†	116 ± 8†	0.245
ICAM-1 (mg/mL)	190.5 ± 7.1	220.4 ± 7.2*	264.4 ± 8.7†	0.031
E-selectin (mg/mL)	18.9 ± 2.0	23.1 ± 2.3†	38.2 ± 2.5†	0.001
C-reactive protein	1.1 ± 0.7	1.6 ± 1.0*	2.4 ± 1.1†	0.028
CK-18 (IU/L) (IU/L) fragments	98 ± 6	136 ± 11	253 ± 12†	0.030
Nitrotyrosine	3.1 ± 3.7	9.9 ± 4.1*	27.4 ± 7.3†	0.002
Met sy (%)	21	36	47†	0.052
Histological steatosis (% hepatocytes)	–	16 ± 7	33 ± 9	0.032
Necroinflammatory grade	–	–	2.1 ± 0.3	–
Fibrosis stage	–	–	1.9 ± 0.4	–
Liver fat score	2.428 ± 0.112	1.563 ± 0.290†	5.114 ± 0.931†	0.039
Liver fat (%)	1.5 ± 1	13 ± 3†	30 ± 6†	0.025
OGTT-derived indexes of glucose homeostasis				
OGIS (mL · minute <sup>-1</sup> · m <sup>-2</sup> )	459.1 ± 16.5	400 ± 11.9*	369 ± 10.2†	0.032
Hepatic extraction (%)	78 ± 4	81 ± 5	70 ± 4*	0.030
Cpgenic Index (CGI) (ng <sub>C-pep</sub> · g <sup>-1</sup> <sub>glucose</sub> )	614 ± 36.1	540 ± 29.5	402 ± 19.3†	0.009
Adaptation Index (ng <sub>C-pep</sub> · g <sup>-1</sup> <sub>glucose</sub> · mL <sup>-1</sup> · m <sup>-2</sup> ) (ng <sub>C-pep</sub> · g <sup>-1</sup> <sub>glucose</sub> · mL <sup>-1</sup> · m <sup>-2</sup> )	281826 ± 15391	216703 ± 12678	148401 ± 10681†	0.007
Muscle insulin sensitivity	0.032 ± 0.009	0.016 ± 0.008*	0.015 ± 0.006*	0.267
Hepatic insulin resistance	738 ± 96	1109 ± 112†	1812 ± 183†	0.011
Oral Fat Load				
IAUC Tg (mg/dL × hour)	124 ± 28	344 ± 49	398 ± 56	0.034
Fasting FFA (μmol/L)	0.47 ± 0.13	1.09 ± 0.22	1.96 ± 0.29	0.112
IAUC FFA (μmol/L × hour)	0.8 ± 0.3	2.5 ± 0.5	4.9 ± 0.9	0.009
Fasting oxidized LDL (uA 234 nm/uA 200 nm × 100)	6.42 ± 1.63	6.44 ± 1.23	7.94 ± 2.59	0.356
IAUC oxidized LDL (uA 234 nm/uA 200 nm × 100 × hour)	1.29 ± 0.51	4.29 ± 1.25	8.02 ± 1.93	0.001

Data are presented as mean ± standard error of the mean. Differences between groups were analyzed by analysis of variance for normal variables; otherwise, the Mann-Whitney test was used for nonparametric variables. Normality was evaluated by Shapiro-Wilk test. Fisher or chi-squared test were used to compare categorical variables, as appropriate. Differences were considered statistically significant at  $P < 0.05$ .

BP, blood pressure; FFA, free fatty acids; HDL-C, high-density lipoprotein cholesterol; IAUC, incremental area under the curve; ICAM-1, intercellular adhesion molecule-1; LDL-C, low-density lipoprotein cholesterol; met sy, metabolic syndrome; OGIS, oral glucose insulin sensitivity index; Tg, triglyceride; total C, total cholesterol.

CGI (Cpgenic index) =  $\Delta C - \text{peptide}_{30'} / \Delta \text{glucose}_{30'}$  during the OGTT; Adaptation Index was computed by multiplying CGI × OGIS. Hepatic extraction is the percent secreted insulin extracted by the liver.

\* $P < 0.05$  versus controls; † $P < 0.01$  versus controls.

NAFLD with a sensitivity, specificity, and positive and negative likelihood ratio of 0.93, 0.80, 4.63, and 0.09, respectively.

The AUROC of CK-18 for NASH was 0.83 (95% CI: 0.80–0.90). A cutoff of 246 IU/L for CK-18 individuated NASH with a sensitivity, specificity, and positive and negative likelihood ratio of 0.78, 0.88, 6.65, and 0.25, respectively.

On multiple regression analysis, liver fat equation independently correlated with hepatic insulin resistance ( $\beta = 0.52$ ; 95% CI: 0.48–0.56,  $P = 0.005$ ) and with indexes of pancreatic  $\beta$ -cell function (for CGI:  $\beta = -0.43$ ; 95% CI: 0.48–0.56,  $P = 0.01$ ; for AI:  $\beta = -0.46$ ; 95% CI: 0.42–0.51,  $P = 0.009$ ) in the whole sample. Liver fat equation also independently predicted plasma C-reactive protein ( $\beta =$

0.40; 95% CI: 0.37–0.44,  $P = 0.02$ ), nitrotyrosine ( $\beta = 0.41$ ; 95% CI: 0.38–0.46,  $P = 0.02$ ), E-selectin ( $\beta = 0.49$ ; 95% CI: 0.45–0.54,  $P = 0.006$ ), and postprandial triglyceride ( $\beta = 0.42$ ; 95% CI: 0.39–0.46,  $P = 0.02$ ) and oxidized low-density lipoprotein ( $\beta = 0.40$ ; 95% CI: 0.38–0.45,  $P = 0.03$ ) responses to the fat load.

Histological steatosis, inflammatory grade, and fibrosis stage were not independently related to the abovementioned parameters.

In conclusion, liver fat equation and CK-18 accurately individuated the presence and severity of liver fat infiltration and the presence of NASH in our cohort of nondiabetic subjects. Most importantly, liver fat equation was tightly related to validated predictors of increased cardiometabolic risk in both healthy and NAFLD subjects. The clini-

cal significance of liver fat equation may thus go beyond hepatic fat content estimation and this easy-to-calculate index may aid in predicting individual cardio-metabolic risk of patients with NAFLD. Our cross-sectional findings warrant prospective confirmation in independent large cohorts.

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## Serum Markers of Hepatocyte Apoptosis: Current Terminology and Predictability in Clinical Practice

### To the Editor:

We read with interest the article by Feldstein et al.,<sup>1</sup> in which they validated the use of cytokeratin-18 (CK-18) fragment serum levels as a noninvasive biomarker for the diagnosis of nonalcoholic steatohepatitis (NASH) in a large cohort of adults with biopsy-proven nonalcoholic fatty liver disease (NAFLD). According to their conclusions, this test can be reliably used to diagnose NASH among patients with suspected NAFLD in clinical practice. This is an important study, because it confirms the significant associations between high CK-18 fragment levels and NASH reported in previous smaller studies in other cohorts of adults<sup>2,3</sup> or even children with NAFLD.<sup>4</sup> However, we would like to draw your attention to two issues: terminology and predictability.

The "Keratin Nomenclature Committee" recently established the new consensus nomenclature for mammalian keratin genes and proteins,<sup>5</sup> which is based on and extends the first comprehensive keratin nomenclature developed back in 1982.<sup>6</sup> According to this new nomenclature, which is in agreement with the nomenclature of the Human Genome Organisation (HUGO) for both gene and protein names,<sup>5</sup> it is suggested the term "keratin" be used instead of "cytokeratin", because it covers all intermediate filament-forming proteins with specific physicochemical properties produced in any vertebrate epithelia.<sup>5</sup> Thus, the term "keratin-18 (K-18)" is now more appropriate than "cytokeratin-18 (CK-18)" for all manuscripts.

K-18 and keratin-8 (K-8) are the only cytoplasmic intermediate filaments of hepatocytes but are not hepatocyte-specific, because they are also expressed in most simple epithelial cells including bile duct cells.<sup>7</sup> K-18 fragment serum levels, which are increasingly used as a biomarker of hepatocyte apoptosis, are also not liver-specific, because these levels may be elevated in patients with epithelial tumors.<sup>8</sup> Moreover, this marker is not specific for NASH, because it is increased in several liver diseases with ongoing necroinflammation and fibrosis, such as chronic hepatitis C or B.<sup>9,10</sup>

The specificity issues are substantially limited if the test is used in patients with probable NAFLD. However, even in such patients followed in specialized centers, its diagnostic accuracy does not seem to be excellent. In the study by Feldstein et al.,<sup>1</sup> the area under the receiver operating characteristic (AUROC) curve for NASH diagnosis was

0.83 (not excellent) with sensitivity and specificity values of 75% and 81% or 65% and 92% for cutoff values of 246 or 292 U/L, respectively. In 58 adult patients with NAFLD studied in our center, K-18 fragment levels offered an AUROC curve of 0.87 and sensitivity and specificity values of 60% and 93%, respectively, for a cutoff of 250 U/L (G. V. Papatheodoridis, unpublished data). Similar findings for variable cutoff values were previously reported by others with the best results reported in the first relevant study.<sup>2,3</sup> Thus, measurement of K-18 fragment levels will probably be helpful in the noninvasive diagnosis of NAFLD, particularly in cases with rather high levels. The specificity issues should be restricted by ensuring the NAFLD diagnosis, but a decision may not be easy in a large proportion of NAFLD cases with K-18 values of <300 U/L, and particularly <200 U/L.

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